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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 220, Revision 2 (FGE.220Rev1): α,β-Unsaturated ketones and precursors from chemical subgroup 4.4 of FGE.19: 3(2H)-Furanones[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

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ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of 10 flavouring substances from subgroup 4.4 of FGE.19 in the Flavouring Group Evaluation 220 (FGE.220). FGE.220 is subdivided into two subgroups (subgroup 4.4a containing [FL-no: 13.089, 13.117, 13.119, 13.157 and 13.175] and subgroup 4.4b containing [13.010, 13.084 and 13.085, 13.099 and 13.176]. For both subgroups the Panel concluded that the genotoxicity alert could not be ruled out based on the data available and accordingly additional genotoxicity data were requested. In FGE.220, Revision 1, the Panel concluded, based on new submitted data, that for the substances in subgroup 4.4b there is no concern for genotoxicity. The Flavour Industry has now provided additional genotoxicity studies for two representative substances of subgroup 4.4a, 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] and 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175]. Based on the new data the Panel concluded that 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] does not give rise to concern with respect to genotoxicity. For 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] the concern for genotoxicity could not be ruled out and therefore the Panel requests a repetition of the submitted micronucleus study in the presence of S9-mix applying the same conditions and possibly in addition modified conditions, or a combined *in vivo* micronucleus and Comet assay, including analysis of liver. This is also applicable to 2,5-dimethyl-4-methoxyfuran-3(2H)-one [FL-no:13.089] and 2,5-dimethyl-4-ethoxyfuran-3(2H)-one [FL-no:13.117], which are covered by the representative substance 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no:13.175]. The Flavour Industry has informed that 5-methylfuran-3(2H)-one [FL-no: 13.157] is not in common use in the flavour industry and is no longer supported.

[1] On request from European Commission, Question No EFSA-Q-2013-00201, EFSA-Q-2013-00202, EFSA-Q-2013-00203, EFSA-Q-2013-00204, adopted on 25 September 2013.

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KEY WORDS

alpha,beta-unsaturated ketones, 3(2H)-furanones, flavouring substances, safety evaluation

SUMMARY

Following a request from European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was asked to evaluate 10 flavouring substances in Flavouring Group Evaluation 220 (FGE.220) using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

Flavouring Group Evaluation 220 (FGE.220) concerned 10 substances, corresponding to subgroup 4.4 of FGE.19. The 10 substances are α,β-unsaturated 3(2H)-furanones [FL-no: 13.010, 13.084, 13.085, 13.089, 13.099, 13.117, 13.119, 13.157, 13.175 and 13.176]. The substances were further subdivided into two subgroups as five [FL-no: 13.089, 13.117, 13.119, 13.157 and 13.175] of the 10 substances can only exist as α,β-unsaturated ketones (subgroup 4.4a) while in the other five substances [13.010, 13.084 and 13.085, 13.099 and 13.176], the α,β double bond can be involved in keto-enol tautomerism (subgroup 4.4b).

For both groups the Panel concluded that the genotoxicity alert could not be ruled out based on data available at that time, and accordingly additional genotoxicity data were requested for both groups. The additional information should be based on specific data requested in FGE.220 and performed on representative substances selected from both groups.

Revision 1 of FGE.220 (FGE.220Rev1) concerned the evaluation of additional data submitted by Industry in response to the requested genotoxicity data in FGE.220 on the representative substance for subgroup 4.4b, 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010]. The Panel concluded that for the substances [13.010, 13.084 and 13.085, 13.099 and 13.176] in subgroup 4.4b there is no concern for genotoxicity, and these substances were accordingly evaluated through the Procedure in FGE.99.

The present revision of FGE.220 (FGE.220Rev2), concerns the evaluation of additional data submitted by Industry in response to requested genotoxicity data in FGE.220 on representative substances for subgroup 4.4a. The Flavour Industry has informed that one of the representative substances, 5-methylfuran-3(2H)-one [FL-no: 13.157], is not in common use in the flavour industry and is no longer supported. As an alternative substance for testing within this subgroup, the Flavour Industry had proposed the structurally related substance 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119]. Accordingly, additional genotoxicity data have now been submitted for 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] and 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175].

These data have been examined by the Panel which concluded that 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] does not give rise to concern with respect to genotoxicity and can accordingly be evaluated using the Procedure. For 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] the concern for genotoxicity could not be ruled out and therefore the Panel requests a repetition of the submitted micronucleus study in the presence of S9-mix applying the same conditions and possibly in addition modified conditions, or a combined *in vivo* micronucleus and Comet assay, including analysis of liver. This is also applicable to 2,5-dimethyl-4-methoxyfuran-3(2H)-one [FL-no:13.089] and 2,5-dimethyl-4-ethoxyfuran-3(2H)-one [FL-no:13.117] which are covered by the representative substance 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no:13.175].

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Background as provided by The European Commission

The use of flavouring is regulated under Regulation (EC) No 1334/2008[4] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of article 9(a) of this Regulation an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

EFSA concluded that a genotoxic potential of five 3(2H)-furanones, corresponding to subgroup 4.4a of FGE.19 in the present FGE.220, could not be ruled out.

Information on two representative materials has now been submitted by the European Flavour Association. These are 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] and 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175]. This information is intended to cover the re-evaluation of these two substances and of two substances from FGE.19 subgroup 4.4a: 2,5-dimethyl-4-methoxyfuran-3(2H)-one [FL-no: 13.089] and 2,5-dimethyl-4-ethoxyfuran-3(2H)-one [FL-no: 13.117].

The commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

Terms of reference as provided by The European Commission

The European Commission requests the European Food Safety Authority to carry out a safety assessment on the following four flavouring substances: 2,5-dimethyl-4-methoxyfuran-3(2H)-one [FL-no: 13.089], 2,5-dimethyl-4-ethoxyfuran-3(2H)-one [FL-no: 13.117], 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] and 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] in accordance with Commission Regulation (EC) No 1565/2000.

History of FGE.19

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a

4 Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Official Journal of the European Communities 31.12.2008, L 354/34-50.

5 EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Communities 2.10.2012, L 267, 1-161.OJ L 267, 2.10.2012, p. 1.

6 Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. Official Journal of the European Communities 19.7.2000, L 180, 8-16.

(quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these α,β-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. The Panel decided that 11 subgroups (1.1.2, 1.1.3, 1.1.4, 2.4, 2.6, 2.7, 3.1, 3.3, 4.1, 4.2 and 4.4) of FGE.19 (EFSA, 2008a) should be further examined to determine whether evaluation through the Procedure is feasible. Corresponding to these 11 subgroups, 11 Flavouring Group Evaluations (FGEs) were established (FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220). If the Panel concludes for any substances in these 11 FGEs that they cannot be evaluated using the Procedure then it has to be decided if there is a safety concern for certain substances or if additional data are required in order to finalise the evaluation. If the Panel concludes that a genotoxic potential can be ruled out for the substances, they will be merged with structurally related substances in other FGEs and evaluated using the Procedure.

To ease the data retrieval of the large number of structurally related α,β-unsaturated substances in the different subgroups for which additional data are requested, EFSA has worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise, an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavouring Industry has now submitted additional data and the present revision of FGE.216 concerns the evaluation of these data requested on genotoxicity.

ASSESSMENT

1. History of the Evaluation of the Substances in the present FGE

EFSA considered in FGE.220 subgroup 4.4 of FGE.19 (EFSA, 2008a). Subgroup 4.4 consists of 10 α,β-unsaturated 3(2H)-furanones, which have been further subdivided into two groups 4.4a and 4.4b based on chemical structures (Table 2). For both groups the Panel concluded that the genotoxicity alert could not be ruled out based on data available at that time, and accordingly additional genotoxicity data were requested for both groups. The additional information should be based on specific data requested in FGE.220 and performed on representative substances selected from both groups (EFSA, 2008c).

In the EFSA Opinion "List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing" (EFSA, 2008c), representative flavouring substances have been selected for subgroups 4.4a and 4.4b, corresponding to FGE.220, for which additional data on genotoxicity were requested, according to the Opinion of the Panel on the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19" (EFSA, 2008b).

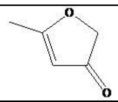
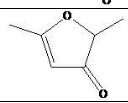
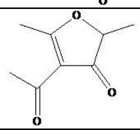
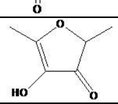
Revision 1 of FGE.220 (FGE.220Rev1) concerned the evaluation of additional data submitted by Industry in response to the requested genotoxicity data in FGE.220 on the representative substance for subgroup 4.4b, 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010]. The description and evaluation of these data are cited from FGE.220Rev1 in Section 5 in the present Revision 2 of FGE.220 (FGE.220Rev2).

FGE	Adopted by EFSA	Link	No. of Substances
FGE.220	29 january 2009	http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902503180.htm	10
FGE.220Rev1	30 September 2010	http://www.efsa.europa.eu/en/efsajournal/pub/1841.htm	10
FGE.220Rev2			9

The present Revision 2 of FGE.220 (FGE.220Rev2), concerns the evaluation of additional data submitted by Industry in response to requested genotoxicity data in FGE.220 on representative substances for subgroup 4.4a (See Table 1). The Flavour Industry has informed that one of the representative substances, 5-methylfuran-3(2H)-one [FL-no: 13.157], is not in common use in the Flavour Industry and is no longer supported. As an alternative substance for testing within this subgroup, the Flavour Industry had proposed the structurally related substance of subgroup 4.4a, 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119]. Accordingly, additional genotoxicity data have been submitted on 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] and 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] (Table 1).

Table 1: Representatives selected by EFSA for Subgroup 4.4 of FGE.220

Representatives selected by EFSA for Subgroup 4.4 of FGE.220 (EFSA, 2008bc)

Subgroup	FL-no	Register name for representatives	Structural formula
4.4a	13.157	5-Methylfuran-3(2H)-one Not supported any longer by EFFA and deleted from the Union List	
	13.119	2,5-Dimethylfuran-3(2H)-one New representative substance suggested by EFFA	
	13.175	4-Acetyl-2,5-dimethylfuran-3(2H)-one	
4.4b	13.010	4-hydroxy-2,5-dimethylfuran-3(2H)-one	

Sections 2-4 report the same information that was present in the earlier versions of FGE.220.

Section 5 describes additional data submitted for subgroup 4.4b in FGE.220Rev1.

Section 6 describes new information on representative substances of subgroup 4.4a.

2. Presentation of the Substances in Flavouring Group Evaluation 220

2.1. Description

The present Flavouring Group Evaluation 220 (FGE.220) concerns 10 substances, which are presented in Table 2. The 10 substances correspond to subgroup 4.4 of FGE.19 (EFSA, 2008a). These substances are all α,β-unsaturated 3(2H)-furanones [FL-no: 13.010, 13.084, 13.085, 13.089, 13.099, 13.117, 13.119, 13.157, 13.175 and 13.176]. Five of the 10 substances can only exist as ketones [FL-no: 13.089, 13.117, 13.119, 13.157 and 13.175] (subgroup 4.4a). In the remaining five substances, the α,β-double bond can be involved in keto-enol tautomerism as such [FL-no: 13.010, 13.084 and 13.085] or after hydrolysis of the ester moiety [13.099 and 13.176] (subgroup 4.4b).

A summary of their current evaluation status of both subgroups 4.4a and 4.4b by the JECFA is given in Table 3 (JECFA, 2006).

The α,β-unsaturated aldehyde and ketone structures are considered by the Panel to be structural alerts for genotoxicity (EFSA, 2008a). Accordingly the available data on genotoxic or carcinogenic activity for the ten ketones in FGE.220 were considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on the ketones in the present FGE. The 10 alpha,beta-unsaturated ketones and their (Q)SAR predictions are described and summarised in Table 4.

3. Toxicity

3.1. (Q)SAR Predictions

In Table 4 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS Local Model-Ames test, DTU-NFI MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), -Chromosomal aberration test in Chinese hamster lung cells (CHL), and -Mouse lymphoma test) are presented.

For none of the candidate substances in this FGE a prediction was obtained with the ISS Local Model for gene mutations in *Salmonella* TA100, as all substances were out of domain. The DTU-NFI MultiCase models for mutagenicity predicted negative (no genotoxic potential) in the Ames test for all 10 substances, and also for three substances (all three in subgroup 4.4b) in the Mouse lymphoma assay. For one substance [FL-no: 13.157] from subgroup 4.4a, a positive response in the Mouse lymphoma assay was predicted. The other candidate substances were out of domain. All but four substances (from subgroup) were out of domain for both the Chromosomal aberration CHO and CHL models. The four substances from subgroup 4.4b were in the domain of the Chromosomal aberrations CHL model and for these four the application of the model resulted in a negative prediction.

It is concluded that these models, except for the negative predictions for the substance in the DTU-NFI MultiCASE model for Ames test, do not seem to generate a reliable and reproducible pattern of predictions for this group. Negative predictions in mammalian cells were only available for four of the substances in subgroup 4.4b (Furan-3(2H)-ones in which the alpha,beta double bond can be involved in keto-enol tautomerism). One positive prediction was available for genotoxic activity in mammalian cells for a substance in subgroup 4.4a (Furan-3(2H)-ones).

3.2. Carcinogenicity Studies

A carcinogenicity study with chronic exposure is available for one substance in subgroup 4.4b

In an OECD Guideline 451- and GLP-compliant study, groups of 60 male and 60 female Sprague-Dawley rats were fed diets containing 0 (controls), 100, 200 or 400 mg 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] per kg body weight (bw) per day for two years. Mean body weights and body weight gains of male and female rats exposed to 400 mg 4-hydroxy-2,5-dimethyl-3(2H)-furanone/kg bw/day were decreased compared to those of the controls in the last part of the study. No neoplasms or non-neoplastic lesions were attributed to exposure to 4-hydroxy-5-dimethyl-3(2H)-furanone. The NOAEL was 200 mg/kg bw/day (Kelly and Bolte, 2003).

The Panel concluded that the study on 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] was valid and did not show a carcinogenic potential in rats.

Study validation and results are presented in Table 5.

3.3. Genotoxicity Studies

Genotoxicity studies are available for four of the candidate substances in FGE.220, as summarised in Tables 6 and 7.

<u>Subgroup 4.4a (Furan-3(2H)-ones)</u>

For one substance in subgroup 4.4a (2,5-dimethyl-3(2H)-furanone [FL-no: 13.119]), no mutagenic activity was observed in *S. typhimurium* in a valid assay. No experimental data were available for any of the other substances in this subgroup.

<u>Subgroup 4.4b (Furan-3(2H)-ones in which the alpha,beta double bond can be involved in keto-enol tautomerism).</u>

For three substances with available genotoxicity studies, which belong to subgroup 4.4b, the following results have been reported:

4-Hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010]

For 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] publications on *in vitro* and *in vivo* studies are available. In three studies the potential of the test substance to induce gene mutations in *S. Typhimurium* was studied. The substance was found positive in two valid studies and in one study with limited validity. The substance did not cause gene mutations in a valid study in *Escherichia coli* WP2 uvrA$^-$. It was also observed that the substance caused DNA repair in a less relevant bacterial test and single strand breaks in purified DNA.

All *in vivo* studies provided indications for a genotoxic potential. Two studies showing micronucleus formation in peripheral blood cells were considered valid (Hiramoto et al., 1996b; Hiramoto et al., 1998); in a third study similar evidence but of limited validity was obtained (Xing et al., 1988). The latter authors also reported an increase in sister chromatid exchanges (SCE) in mouse bone marrow, but the validity of that observation could not be assessed. In addition, this endpoint is of questionable relevance for the assessment of genotoxicity.

In addition to the genotoxicity observed in somatic cells, three studies provided evidence for genotoxicity in germ cells.

The evidence of chromosome aberration induction in mouse germ cells provided in the study by Xing et al. (1988) is poor because it is essentially based on an increase of premature disjunction of sex chromosomes and autosomes at metaphase I. This effect could be considered at most an alert of possible subsequent missegregation events; even so, data have been published (Liang and Pacchierotti, 1988) showing the lack of correlation between univalents at metaphase I and aneuploidy at metaphase II.

Tian et al. (1992) reported an induction of SCE in spermatogonia. Incomplete information is given on the experimental protocol. There is a dose-dependent increase of SCE/cell, with each dose group significantly higher than the negative control. For these reasons, these data seem to be convincing although obtained on a small (3) number of animals/group. The relevance of SCE in spermatogonia as an indicator of heritable genetic damage is limited.

In the same paper Tian et al. (Tian et al., 1992) reported the induction of micronuclei in early sperm cells. This test measures the induction of DNA lesions in preleptotene spermatocytes that can lead to breaks and fragments several days later, at the first or second meiotic division. The test has not been standardised and validated for routine regulatory application, but has been conducted by more than one laboratory in the world with consistent results. The study seems adequately performed. Staining with Giemsa is not optimal and does not allow to distinguish among phases of spermatid differentiation as recommended by the guidelines (Russo, 2000). However, this drawback could hardly produce an overestimation of the effect, more likely, if any, an underestimation.

4-Hydroxy-5-methylfuran-3(2H)-one [FL-no: 13.085] and 2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone [FL-no: 13.084]

Reverse mutations were also observed in *S. typhimurium* TA100, but not TA98 with 4-hydroxy-5-methylfuran-3(2H)-one [FL-no: 13.085] and with 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone [FL-no: 13.084]. The other strains were not tested. The same substances could induce single strand breaks in purified DNA. With 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone [FL-no: 13.084] also induction of micronuclei in peripheral erythrocytes was observed in two valid *in vivo* assays.

Mechanistic data

For the substances in subgroup 4.4b also mechanistic studies were carried out with [FL-no: 13.010, 13.084 and 13.085], all of which were considered valid. These substances were identified as Maillard reaction products in soy sauce. When the substance [FL-no: 13.085] was incubated with supercoiled pBR 322 plasmid DNA, single strand breaks were observed at pH 4.4, but not at pH 7.4. When a spin trap was also present, formation of hydroxy radicals together with a carbon-centered radical could be demonstrated. Subsequent addition of superoxide dismutase and catalase inhibited the DNA breaking showing involvement of hydrogen peroxide. Potassium iodide, mannitol, sodium azide and ethanol were also inhibitory to the DNA breaking showing involvement of hydroxy radicals. Spin trapping agents and thiol compounds and metal chelators also effectively inhibited the breaking of DNA (Hiramoto et al., 1996a). Similar studies (Hiramoto et al., 1996b; Li et al., 1998) were carried out with [FL-no: 13.010 and 13.084] with the same results and it was also demonstrated that these substances are capable to reduce Fe^{3+} at neutral or alkaline pH (Li et al., 1998).

Study results and comments on study validity are presented in Table 6 and 7.

3.4. Conclusion on Genotoxicity and Carcinogenicity – Text taken from FGE.220[7] (EFSA, 2009)

Apart from the negative predictions for the substances in the DTU-NFI MultiCASE model for the Ames test, the (Q)SAR models do not seem to generate a reliable and reproducible pattern of predictions on the genotoxicity for the substances in this FGE.

For one substance in subgroup 4.4a (2,5-dimethyl-3(2H)-furanone [FL-no: 13.119]) no mutagenic activity was observed in *S. typhimurium* in a valid assay. This study result is insufficient to reach a conclusion as to the (absence) of genotoxicity for this subgroup.

With several substances in subgroup 4.4b indications have been obtained in *in vitro* studies that the genetic damage they cause is related to the generation of reactive oxygen species as a result of redox cycling in combination with metal ions present in the media. The valid positive *in vivo* data were obtained with high dose levels that may be anticipated to have exhausted the anti-oxidant capacity of the target cells. This, in combination with the absence of carcinogenicity observed in a valid carcinogenicity study in rats with one of the substances [FL-no: 13.010], which was tested positive in the genotoxicity assays, takes away a concern for genotoxic events resulting in carcinogenicity in somatic cells.

For two of the studies in which genotoxic effects were observed in germ cells *in vivo* the studies had limited validity and/or address endpoints that may have limited relevance for the assessment of genotoxic potential. The Panel noted that a positive result was obtained in a micronucleus study in

[7] The conclusion in Section 3.4 is cited from the previous version of the present FGE, FGE.220. This conclusion is the basis for the request of additional genotoxicity data in FGE.220.

early sperm cells. However, a micronucleus test does not discriminate between aneuploidy and chromosomal breakage. The observed effects in the germ cells could be the result of the malsegregation of chromosomes which is generally considered a thresholded event. They may alternatively be the result of the (thresholded) generation of reactive oxygen species.

4. Conclusions – Text taken from FGE.220[8] (EFSA, 2009)

For the substances in subgroup 4.4a [FL-no: 13.089, 13.117, 13.119, 13.157 and 13.175], the Panel considered that presently the available data on genotoxicity are too limited to evaluate these substances through the Procedure. Additional studies are needed as outlined in the Genotoxicity Test Strategy for Substances belonging to Subgroups of FGE.19 (EFSA, 2008b).

For the substances in subgroup 4.4b [FL-no: 13.010, 13.084, 13.085, 13.099 and 13.176], evidence for genotoxicity was obtained *in vitro* and *in vivo*. Evidence is available from *in vitro* studies that the genotoxicity of the candidate substances in this subgroup may be caused by indirect (thresholded) mechanisms of action (in particular generation of reactive oxygen species). The concern for carcinogenicity is alleviated, since one of the substances, for which positive genotoxicity data in mice were obtained, was not carcinogenic in a valid chronic assay in rats. Therefore, no further genotoxicity tests in somatic cells are required. However, some evidence was also available that this substance might elicit genotoxic effects in germ cells, which theoretically may result in reduced reproductive capacity or in inheritable genetic damage. Reduced reproductive capacity and inheritable genetic damage are toxicological endpoints which differ from carcinogenicity and therefore, the negative results for the carcinogenicity study cannot be used to overrule this concern. Also it is not clear if (and if so to what extent) the thresholded mechanism mentioned above would be relevant for genotoxic effects in the germ cells. Therefore, the Panel concluded that presently these five substances cannot be evaluated through the Procedure.

The Panel recognised that the studies which provided indications for germ cell genotoxicity are of limited validity. For that reason a robust GLP-controlled cytogenetic investigation in mouse spermatocytes according to the OECD Guideline 483 is requested.

5. Additional data submitted by Industry for Subgroup 4.4b (FGE.220Rev1)

In response to the EFSA request in FGE.220, of a cytogenetic study in mouse spermatocytes (OECD TG 483), Industry has submitted the following data:

- Two-year carcinogenicity bioassay in rats with a substance coded ST 07 C99 (this is the study on [FL-no: 13.010] by Kelly & Bolte, 2003);
- Oral male fertility study of FURANEOL = 4-Hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] (test article code ST17C07) in rats (Sloter, 2008);
- Oral micronucleus assay in bone marrow cells of the mouse with NEOFURANEOL (no identification of this substance is available) (Honarvar, 2008b);
- Mouse lymphoma (TK) specific locus mutation assay with compound 0478/1 (Ross & Harris, 1979a).

5.1. Evaluation of Additional Data for Subgroup 4.4b (FGE.220Rev1)

The Panel noted that among the studies submitted by Industry only the rat fertility study, which includes also the analysis of dominant lethals, is considered relevant for the specific EFSA request.

[8] The conclusion in Section 4 is cited from the previous version of the present FGE, FGE.220. This conclusion is the basis for the request of additional genotoxicitydata in FGE.220.

The 2-year carcinogenicity bioassay in rats by Kelly and Bolte (Kelly and Bolte, 2003) was already evaluated by the Panel in the previous version of this FGE (Section 3.2 (Table 5)). It was considered as a valid, negative study, however not relevant for the evaluation of possibly inheritable damage. Also the mouse bone marrow micronucleus assay with neofuraneol (Honarvar, 2008b) and the *in vitro* mouse lymphoma TK assay (Ross and Harris, 1979a) are considered not relevant to clear the concern for possible inheritable damage. Furthermore, an adequate identification of the test substance Neofuraneol was not possible, due to incomplete reporting. For these reasons these three studies will not be further considered in this section.

Oral Male Fertility Study of 4-Hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] in Rats (Sloter, 2008)

The objective of this study, performed according to ICH Guideline 4.1.1 (ICH, 1996) under GLP, was to determine the potential effects of 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] on mating, fertility and gonadal function in male rats with two separate mating trials. 4-Hydroxy-2,5-dimethylfuran-3(2H)-one was administered by gavage once daily to three groups of 25 male Crl:CD(SD) rats. Dosage levels were 100, 500 and 1000 mg/kg bw/day. A concurrent control group of 25 males received the vehicle (propylene glycol) on a comparable regimen. The first mating (Phase I), following 2 weeks of male administration, using untreated females, was conducted to detect potential elicitation of early genotoxic effects on the embryo with reduced risk of test-article related deficiencies in mating or fertility. The second mating (Phase II), following 9 weeks of male dose administration, was conducted following male exposure throughout a complete spermatogenic cycle using a second set of untreated females.

There was no test-article related mortality noted in this study. A slightly lower mean body-weight gain was noted in the 1000 mg/kg/day group when evaluated for the overall treatment period. No test-article related effects on male reproductive performance were observed at 100, 500 and 1000 mg/kg/day when males were mated with Phase I or Phase II females. In particular, there were no effects on spermatogenic endpoints (mean testicular and epididymal sperm numbers, sperm production rate, motility and morphology, reproductive organs or macroscopic findings) at any of the doses tested. The mean percentage of sperm with abnormal morphology (separated head and flagellum) was slightly higher in the 500 and 1000 mg/kg/day groups; however, this was primarily attributed to a single male in the respective groups and therefore not considered test-article related. The number of females mated and the number of pregnant females was comparable to controls. Uterine examination was performed for both Phase I and Phase II females. The analysis of embryonic data (corpora lutea, implantation sites, viable embryos, dead embryos, early resorptions, late resorptions, total resorptions, post- and pre-implantation losses) did not reveal dominant lethal effects. The study does not indicate a potential of 4-hydroxy-2,5-dimethylfuran-3(2H)-one [FL-no: 13.010] to affect male fertility. This study can be considered to be equivalent to an OECD 478 Dominant Lethal assay. The Dominant Lethal assay has been recommended as follow-up study in case of positive results in the OECD TG 483 (Eastmond et al., 2009). On this basis the Panel considers it acceptable to substitute the requested study according to OECD Guideline 483 with the Dominant Lethal test.

Study results and comments on study validity are presented in Table 8.

5.2. Conclusion on Additional Data for Subgroup 4.4b (EFSA, FGE.220Rev1)

The results of a valid rat fertility and dominant lethal study have shown that 4-hydroxy-2,5-dimethylfuran-3(2H)-one is unable to induce both adverse effects on male rat reproductive capacity and dominant lethality. On this basis the Panel concludes that for this substance there is no concern for its potential to induce heritable genetic damage or adverse effects on male reproductive capacity. Accordingly, the substances in subgroup 4.4b of FGE.19 [FL-no: 13.010, 13.084, 13.085, 13.099 and 13.176] can be evaluated using the Procedure.

Since no data were submitted to further evaluate the genotoxic potential of the substances in subgroup 4.4a, the Panel in FGE.220Rev1 maintained its position that for this subgroup additional data on genotoxicity are needed.

6. Additional data submitted by Industry for Subgroup 4.4a

In response to the EFSA request in FGE.220 for additional genotoxicity data for subgroup 4.4a the Flavour Industry (IOFI, 2012) has submitted *in vitro* genotoxicity data on:

- 2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119] (Ames test and *in vitro* micronucleus assay)

- 4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] (Ames test and *in vitro* micronucleus assay)

6.1. *In vitro* Genotoxicity Studies for Subgroup 4.4a

Bacterial mutation assays

2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119]

2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119] was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence or presence of S9-mix (Sokolowski, 2007) in a GLP study and according to OECD Test Guideline 471. In the first experiment the concentrations tested were 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg/plate, and plate incorporation methodology was used. In the second experiment the concentrations were 33, 100, 333, 1000, 2500 and 5000 μg/plate of 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119], and treatments in the absence and in the presence of S9-mix used the pre-incubation method. No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation. The solvent control data reported for strain TA102 in the absence of S9-mix, indicated slightly increased numbers of revertant colony numbers (538 ± 28) compared to historical controls (407.1 ± 78.3). Since the effect is small in the control, the effect is considered by the study director to be based upon biologically irrelevant fluctuations in the number of colonies. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] in any of the strains, either in the absence or presence of S9-mix.

4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175]

4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Bowen, 2011) in a GLP study and according to OECD Test Guideline 471. In the first experiment the concentrations were 0.32, 1.6, 8, 40, 200, 1000 and 5000 μg/plate of 4-acetyl-2,5-dimethylfuran-3-(2H)-one [FL-no: 13.175] and the plate incorporation methodology was used. Slight thinning of the background lawn was observed at 5000 μg/plate for all test strains in the absence and presence of S9-mix. In the second experiment the concentrations were 78.13, 156.3, 312.5, 625, 1250, 2500 and 5000 μg/plate and the treatments in the presence of S9-mix used the pre-incubation method. No clear evidence of toxicity was observed. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] in any of the strains, either in the absence or presence of S9-mix.

Micronucleus Assays

2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119]

2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119] was evaluated in an GLP *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system. Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells, and then treated for 3 hours (followed by 21 hours recovery) with 0, 900, 1000 or 1120 μg/ml of 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] in the absence and in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 12 % and 2 % respectively. In a parallel assay, cells were treated for 24 hours with 0, 900, 1000 and 1120 μg/ml of 2,5-dimethylfuran-3(2H)-one [FL-no: 13.119] in the absence of S9-mix with no recovery period. The top concentration induced 22 % cytotoxicity. There were 2 replicate cultures per treatment, and 1000 binucleate cells per replicate (i.e. 2000 cells per concentration) were scored for micronuclei. Thus the study design complies with current recommendations (OECD Test Guideline 487), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed by increased levels of micronucleated binucleate cells (MNBN) in the presence or absence of S9-mix metabolic activation (Lloyd, 2011).

4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL-no 13.175]

In a similar GLP experiment, human peripheral lymphocytes were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells, and then treated for 3 hours (followed by 21 hours recovery) with 0, 1000, 1250 or 1542 μg/ml of 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] in the absence and in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 20 % and 7 % respectively. In a parallel assay, cells were treated for 24 hours with 0, 400, 600, 900 and 950 μg/ml of 4-acetyl-2,5-dimethylfuran-3(2H)-one [FL-no: 13.175] in the absence of S-9 with no recovery period. The top concentration induced 54% cytotoxicity. There were two replicate cultures per treatment, and 1000 binucleate cells per replicate were scored for micronuclei. Thus the study design complies with current recommendations (OECD Test Guideline 487), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. Initially (following the scoring of 1000 binucleate cells/culture), treatment of cells for 3 hours with a 21 hour recovery period in the presence of S9-mix resulted in mean frequencies of MNBN cells (0.55 %, 0.85 % and 1.25 %, at 1000, 1250 and 1542 μg/ml, respectively) that were significantly higher ($p \leq 0.01$) compared with those observed in concurrent controls (0.20%) at all three concentrations analysed, giving 3 %, 0 % and 7 % reductions in Replication Index, respectively (Annex I, Table 10, Lloyd, 2012). The MNBN cell frequencies exceeded the normal range (0.1% to 1.1 %) only in single cultures at 1250 and 1542 μg/ml (1.2 % and 1.6 %, respectively). It was noted that one of the solvent control replicates fell to 0 %, which is outside of historical control levels and would have impacted the statistical significance. To confirm this result additional 1000 binucleate cells were scored for the vehicle controls “C” and “D” replicate cultures derived from new human blood cultures and an additional 1000 binucleate cells were scored from each of the three test article concentrations analysed, derived from the same human blood culture used in the first experiment. Following the additional scoring, the mean frequencies of MNBN cells were significantly higher but at lower statistical level ($p \leq 0.05$), compared to those observed in the concurrent vehicle controls at the two highest concentrations analysed (1250 and 1542 μg/ml) (Annex I, Table 10, Lloyd, 2012). It was noted that only one culture at 1542 μg/ml (1.25 %) exceeded the normal range. The Panel noted that the additional scoring was conducted with an unjustified and non-homogeneous approach: for the solvent controls the additional 1000 cells were derived from new blood lymphocytes cultures, whereas for the additional scoring of the treated samples, cells were derived from the same blood cultures used in the first experiment. Overall, differently from the authors, the Panel concluded that the results of the *in vitro* micronucleus assay in the presence of S9-mix have to be considered as equivocal instead of negative and therefore the test should be repeated (Lloyd, 2012).

Study results and comments on study validity are presented in Table 9.

6.2. Conclusions on Additional Data Submitted for Subgroup 4.4a

2,5-Dimethylfuran-3(2H)-one [FL-no: 13.119] did not induce mutations in the Ames test and did not induce increased levels of micronuclei in an *in vitro* micronucleus assay with and without metabolic activation. The Panel therefore concluded that [FL-no: 13.119] does not give rise to concern with respect to genotoxicity and accordingly can be evaluated using the Procedure.

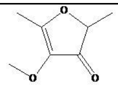
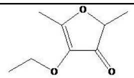
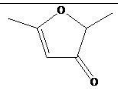
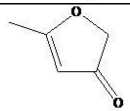
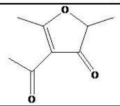
4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL 13.175] did not induce mutations in the Ames test with and without metabolic activation and did not induce increased levels of micronuclei in an *in vitro* micronucleus assay in the absence of S9-mix. However, the results of the micronucleus assay in the presence of S9-mix were considered by the Panel to be equivocal. Therefore, the results of the *in vitro* micronucleus assay should be clarified, e.g. by repetition of the study in the presence of S9-mix applying the same conditions and possibly in addition modified conditions, or by a combined *in vivo* micronucleus and comet assay, including analysis of liver. This is also applicable to 2,5-Dimethyl-4-methoxyfuran-3(2H)-one [FL-no:13.089] and 2,5-Dimethyl-4-ethoxyfuran-3(2H)-one [FL-no:13.117] which are covered by the representative substance 4-Acetyl-2,5-dimethylfuran-3(2H)-one [FL-no:13.175].

Table 2: Specification Summary of the Substances in the Flavouring Group Evaluation 220 (JECFA, 2006)

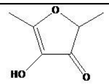
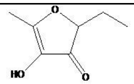
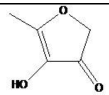
Specification Summary of the Substances in the Flavouring Group Evaluation 220 (JECFA, 2006)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)

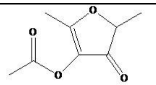
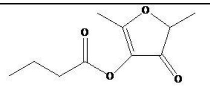
Substances in subgroup 4.4a (Furan-3(2H)-ones)

13.089 1451	2,5-Dimethyl-4-methoxyfuran-3(2H)-one		3664 4077-47-8	Liquid $C_7H_{10}O_3$ 142.15	Insoluble Soluble	61-63 (0.4 hPa) NMR 97 %	1.475-1.481 1.091-1.097
13.117	2,5-Dimethyl-4-ethoxyfuran-3(2H)-one		 65330-49-6	Solid $C_8H_{12}O_3$ 156.18	 1 ml in 1 ml	251 60 95 %	n.a. n.a.
13.119	2,5-Dimethylfuran-3(2H)-one		 11066 14400-67-0	Liquid $C_6H_8O_2$ 112.13	 1 ml in 1 ml	68 (16 hPa) 95 %	1.473-1.479 1.050-1.060
13.157	5-Methylfuran-3(2H)-one		 3511-32-8	Liquid $C_5H_6O_2$ 98.10	 1 ml in 1 ml	59 (13 hPa) 95 %	1.492-1.498
13.175	4-Acetyl-2,5-dimethylfuran-3(2H)-one			Solid $C_8H_{10}O_3$ 154.17	 1 ml in 1 ml	283 34 95 %	n.a. n.a.

Substances in subgroup 4.4b (Furan-3(2H)-ones in which the alpha,beta-unsaturated double bond can be involved in keto-enol tautomerism)

13.010 1446	4-Hydroxy-2,5-dimethylfuran-3(2H)-one		3174 536 3658-77-3	Solid $C_6H_8O_3$ 128.13	Insoluble Soluble	n.a. 78-80 IR 98 %	n.a. n.a.
13.084 1449	2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone		3623 27538-09-6	Liquid $C_7H_{10}O_3$ 142.15	Soluble Soluble	103 (20 hPa) NMR 96 %	1.509-1.514 1.133-1.143
13.085 1450	4-Hydroxy-5-methylfuran-3(2H)-one		3635 11785 19322-27-1	Solid $C_5H_6O_3$ 114.10	Soluble Soluble	n.a. 126-133 NMR 97 %	n.a. n.a.

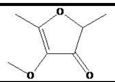
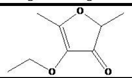
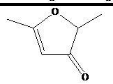
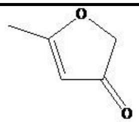
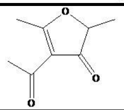
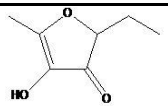
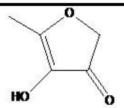
Specification Summary of the Substances in the Flavouring Group Evaluation 220 (JECFA, 2006)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
13.099 1456	4-Acetoxy-2,5-dimethylfuran-3(2H)-one		3797 4166-20-5	Liquid $C_8H_{10}O_4$ 170.17	Slightly soluble Soluble	243 NMR 85 %	1.476-1.480 1.159-1.167
13.176 1519	Furaneyl butyrate		3970	Liquid $C_{10}H_{14}O_4$ 198.22	Insoluble Soluble	287 NMR 93 %	1.467-1.473 1.095-1.103

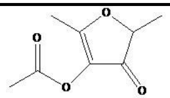
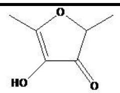
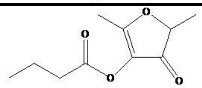
1) Solubility in water, if not otherwise stated.
2) Solubility in 95% ethanol, if not otherwise stated.
3) At 1013.25 hPa, if not otherwise stated.
4) At 20°C, if not otherwise stated.
5) At 25°C, if not otherwise stated.
n.a.: not applicable.

Table 3: Summary of Safety Evaluation Applying the Procedure (Based on Intakes Calculated by the MSDI Approach)

Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2006)

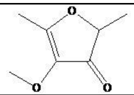
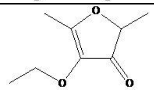
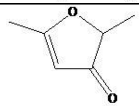
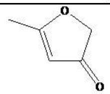
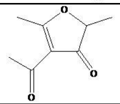
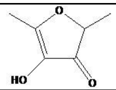
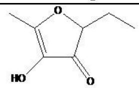
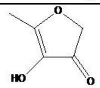
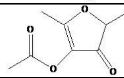
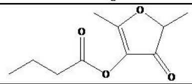
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
Substances in subgroup 4.4a (Furan-3(2H)-ones)							
13.089 1451	2,5-Dimethyl-4-methoxyfuran-3(2H)-one		12 0.7	Class II A3: Intake below threshold	4)	Evaluted in FGE.220 and FGE.220Rev2, additional data required.	
13.117	2,5-Dimethyl-4-ethoxyfuran-3(2H)-one		0.018	No evaluation	Not evaluated by JECFA	Evaluted in FGE.220, additional data required and FGE.220Rev2,	
13.119	2,5-Dimethylfuran-3(2H)-one		1.9	No evaluation	Not evaluated by JECFA	Evaluted in FGE.220, additional data required. Based on new data, evaluted in FGE.220Rev2, genotoxicity concern could be ruled out.	
13.157	5-Methylfuran-3(2H)-one		0.0061	No evaluation	Not evaluated by JECFA	Evaluted in FGE.220, additional data required..	No longer supported by Industry.
13.175	4-Acetyl-2,5-dimethylfuran-3(2H)-one		1.3	No evaluation	Not evaluated by JECFA	Evaluted in FGE.220, additional data required and FGE.220Rev2,	
Substances in subgroup 4.4b (Furan-3(2H)-ones in which the alpha,beta-unsaturated double bond can be involved in keto-enol tautomerism)							
13.084 1449	2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone		203 13	Class II A3: Intake below threshold	4)	Evaluted in FGE.220Rev1, genotoxicity concern could be ruled out. EFSA allocated the substance to Class III. No safety concern at the estimated level of intake based on the MSDI approach.	No safety concern at the estimated level of intake based on the MSDI approach.
13.085 1450	4-Hydroxy-5-methylfuran-3(2H)-one		47.8 0.07	Class II A3: Intake below threshold	4)	Evaluted in FGE.220Rev1, genotoxicity concern could be ruled out. EFSA allocated the substance to Class III. No safety concern at the estimated level of intake based on the MSDI approach.	No safety concern at the estimated level of intake based on the MSDI approach.

Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2006)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
13.099 1456	4-Acetoxy-2,5-dimethylfuran-3(2H)-one		400 8	Class II A3: Intake below threshold	4)	Evaluted in FGE.220Rev1, genotoxicity concern could be ruled out. EFSA allocated the substance to Class III. No safety concern at the estimated level of intake based on the MSDI approach.	No safety concern at the estimated level of intake based on the MSDI approach.
13.010 1446	4-Hydroxy-2,5-dimethylfuran-3(2H)-one		4483 5203	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)	Evaluted in FGE.220Rev1, genotoxicity concern could be ruled out. No safety concern at the estimated level of intake based on the MSDI approach.	No safety concern at the estimated level of intake based on the MSDI approach.
13.176 1519	Furaneyl butyrate		4.2 4	Class III No evaluation		Evaluted in FGE.220Rev1, genotoxicity concern could be ruled out. No safety concern at the estimated level of intake based on the MSDI approach.	No safety concern at the estimated level of intake based on the MSDI approach. Register name to be changed to 4-Butyroxy-2,5-dimethyl-3(2H)-furanone (EFFA, 2012).

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.
2) Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.
3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
4) No safety concern based on intake calculated by the MSDI approach of the named compound.
5) Data must be available on the substance or closely related substances to perform a safety evaluation.

Table 4: QSAR Predictions on Mutagenicity in Five Models for 10 Ketones from Subgroup 4.4

FL-no JECFA-no	Sub-group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
Substances in subgroup 4.4a (Furan-3(2H)-ones)									
13.089 1451	4.4	2,5-Dimethyl-4-methoxyfuran-3(2H)-one		3664 - 4077-47-8	OD*	NEG	OD*	OD*	OD*
13.117	4.4	2,5-Dimethyl-4-ethoxyfuran-3(2H)-one		- - 65330-49-6	OD*	NEG	OD*	OD*	OD*
13.119	4.4	2,5-Dimethylfuran-3(2H)-one		- 11066 14400-67-0	OD*	NEG	OD*	OD*	OD*
13.157	4.4	5-Methylfuran-3(2H)-one		- - 3511-32-8	OD*	NEG	POS	OD*	OD*
13.175	4.4	4-Acetyl-2,5-dimethylfuran-3(2H)-one		- - -	OD*	NEG	OD*	OD*	OD*
Substances in subgroup 4.4b (Furan-3(2H)-ones in which the alpha,beta-unsaturated double bond can be involved in keto-enol tautomerism)									
13.010 1446	4.4	4-Hydroxy-2,5-dimethylfuran-3(2H)-one		3174 536 3658-77-3	OD*	NEG	NEG	OD*	NEG
13.084 1449	4.4	2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone		3623 - 27538-09-6	OD*	NEG	NEG	OD*	NEG
13.085 1450	4.4	4-Hydroxy-5-methylfuran-3(2H)-one		3635 11785 19322-27-1	OD*	NEG	NEG	OD*	NEG
13.099 1456	4.4	4-Acetoxy-2,5-dimethylfuran-3(2H)-one		3797 - 4166-20-5	OD*	NEG	OD*	OD*	OD*
13.176	4.4	Furaneyl butyrate		3970 - -	OD*	NEG	OD*	OD*	NEG

Column 2: Structure group 4.4: α,β-unsaturated ketones.
Column 6: Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD*: out of domain).
Column 7: MultiCase Ames test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 8: MultiCase Mouse Lymphona test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 9: MultiCase Chromosomal aberration in CHO (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 10: MultiCase Chromosomal aberration in CHL (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
* OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological, *etc.*

Table 5: Carcinogenicity Studies

Carcinogenicity Studies

Chemical Name [FL-no]	**Species; Sex No./Group**	**Route**	**Dose levels**	**Duration**	**Results**	**Reference**	**Comments**
4-hydroxy-2,5-dimethylfuran-3(2H)-one [13.010]	Rats; Male, Female 60/sex/group	Diet	0, 100, 200, or 400 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increases in tumour incidences	(Kelly and Bolte, 2003)	Valid (GLP/OECD compliant). The NOAEL was 200 mg/kg bw/day based on reduced mean body weight at the highest dose.

Table 6: Genotoxicity (*in vitro*)

Genotoxicity (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [e]
4-hydroxy-2,5-dimethylfuran-3(2H)-one [13.010]	Reversed mutation	*S. typhimurium* TA1535, TA1537, TA1538,TA100 and TA98	10.0, 33.3, 100.0, 333.3, 1000, 2000, 3300, 4000, 6000, 8000 µg/plate	Positive[a, b]	(Gilroy et al., 1978)	Valid. Unpublished non-GLP study. The report contains sufficient details. Result is considered valid.
	Reversed mutation	*S. typhimurium* TA100 and TA98	0 - 10000 µg/plate	Positive[a, b]	(Hiramoto et al., 1996b)	Valid. Positive in TA100 (+/– S9); negative in TA 98 (+/- S9).
	Reversed mutation	*S. typhimurium* TA100, TA102, TA98 and TA97	500 - 4000 µg/plate	Positive[a, c]	(Xing et al., 1988)	Limited validity. No methodological details, but stated to be performed according to (Maron and Ames, 1983). Some errors reduce the trustworthiness of the paper.
	Reversed mutation	*E. coli* WP2 uvrA$^-$	10.0, 33.3, 100.0, 333.3, 1000, 3300 µg/plate	Negative	(Gilroy et al., 1978)	Valid. Unpublished non-GLP study. The report contains sufficient details. Result is considered valid.
	DNA damage	*B. subtilis* H17 (Rec$^+$) and M45 (Rec$^-$)	20, 40, 60, 80, 120 µg/disc	Positive	(Xing et al., 1988)	Validity cannot be evaluated (Test system with low predictive value for genotoxicity). No methodological details, but stated to be performed according to (Kada et al., 1972).
	DNA strand breaks	pBR322 DNA	2.6 - 780 µmol/l (0.3 - 100 mg/l)	Positive	(Hiramoto et al., 1996b)	Valid. Single strand breaks caused by redox cycling of the substance in combination with metal ions, generating reactive oxygen species.
4-Hydroxy-5-methylfuran-3(2H)-one [13.085]	Reversed mutation	*S. typhimurium* TA100 and TA98	0 - 5000 µg/plate	Positive[a, b]	(Hiramoto et al., 1996a)	Limited validity. Limited due to uncertainty of test substance. Positive in TA100 (+/– S9); negative in TA 98 (+/- S9).
	DNA strand breaks	pBR322 DNA	0 - 900 µmol/l (0 - 103mg/l)	Positive[a, d]	(Hiramoto et al., 1996a)	Valid. Single strand breaks caused by redox cycling of the substance in combination with metal ions, generating reactive oxygen species.
2,5-Dimethyl-3(2H)-Furanone [13.119]	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98,TA100 and TA102,	0 - 5000 µg/plate	Negative	(RCC - CCR, 2007)	Valid. According to current guidelines.
2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone [13.084]	Reversed mutation	*S. typhimurium* TA100 and TA98	0 - 10000 µg/plate	Positive[a, b]	(Li et al., 1998)	Valid. Positive with and without S9 in TA 100; negative in TA98 (+/- S9).
	DNA strand breaks	pBR322 DNA	0 - 2000 µM	Positive[d]	(Li et al., 1998)	Valid. Single strand breaks caused by redox cycling of the substance in combination with metal ions, generating reactive oxygen species.

a: With and without metabolic activation provided by S9 (9000 x g supernatant from rodent liver).
b: Positive results only observed in TA100.
c: Positive results in all strains at the highest dose tested.
d: Only positive without inhibitors of redox cycling and ROS scavengers.
e: Validity of genotoxicity studies:
Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

Table 7: Genotoxicity (*in vivo*)

Genotoxicity (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Reported Result	Reference	Comments [a]
4-hydroxy-2,5-dimethylfuran-3(2H)-one [13.010]	Micronucleus formation	Mouse, bone marrow	Not stated	0, 186, 232 or 309 mg/kg bw	Positive	(Xing et al., 1988)	Limited validity. Important data not given; Reference to methodological description could not be traced.
	Chromosomal aberration	Mouse spermatocytes	Not stated	0, 232, 464 or 928 mg/kg bw	Positive	(Xing et al., 1988)	Limited validity. Important data not given. Reference to methodological description could not be traced. Predominant aberration: malsegregation of chromosomes.
	Sister chromatid exchange	Mouse, bone marrow	Intra-abdominal injection	0, 185, 232, 303 mg/kg	Positive	(Xing et al., 1988)	Validity cannot be assessed. Dose-related increase; statistically significant at all dose levels, but max increase < 2-fold. Effect not adequately specified; very intense exposure to BrdU. Non-validated protocol. Relevance for the evaluation of genotoxicity questionable.
	Sister chromatid exchange	Mouse spermatocytes	Oral (gavage)	200, 400 or 800 mg/kg bw	Positive	(Tian et al., 1992)	Limited validity. Relevance for the evaluation of genotoxicity questionable; non-validated test protocol.
	Micronucleus formation	Mouse early sperm cells	Oral (gavage)	200, 400 or 800 mg/kg bw	Positive	(Tian et al., 1992)	Limited validity. Non-validated test protocol.
	Micronucleus formation	Mouse peripheral blood cells	Gavage	1000, 2000 3000 mg/kg bw	Positive	(Hiramoto et al., 1998)	Valid.
	Micronucleus formation	Male mice peripheral erythrocytes	I.p.	500, 1000, 1500 mg/kg bw	Positive	(Hiramoto et al., 1996b)	Valid.
2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone [13.084]	Micronucleus formation	Mouse peripheral blood cells	Gavage	0, 1000, 2000, and 3000 mg/kg bw	Positive	(Hiramoto et al., 1998)	Valid.
	Micronucleus formation	Male mice peripheral erythrocytes	I.p.	0, 500 and 1000 mg/kg bw	Positive	(Li et al., 1998)	Valid.

a: Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

Table 8: Summary of Additional Genotoxicity Data on 4-hydroxy-2,5-dimethylfuran-3(2H)-one Submitted by Industry

Summary of New Genotoxicity Data Submitted on Subgroup 4.4b (*in vitro* and *in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Reported Result	Reference	Comments [a]
4-hydroxy-2,5-dimethylfuran-3(2H)-one [13.010]	Mouse Lymphoma	L5178Ytk+/- mouse lymphoma cells	-	111, 167, 250, 375 and 750 micrograms/ml	Negative both with and without S9	(Ross and Harris, 1979a)	limited validity. Study not performed according to current guideline. To short treatment and no differentiation between small and large colonies.
	Dominant Lethal assay in a rat fertility study	Dominant lethals in Crl:CD(SD) male rats (25/group)	Oral gavage	100, 500 and 1000 mg/kg bw/day for 2 weeks(Phase I) and 9 weeks (Phase II)	No increase of dominant lethal effects	(Sloter, 2008)	Valid GLP study in accordance with ICH Guideline 4.1.1.

A study by Honarvar (Honarvar, 2008) was also submitted. However due to unknown identity of the tested material, this study is not included in the table.

Table 9: Summary of Additional Genotoxicity Data on 2,5-Dimethylfuran-3(2H)-one and 4-Acetyl-2,5-dimethylfuran-3(2H)-one Submitted by Industry

Summary of New Genotoxicity Data Submitted on Subgroup 4.4a (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Dose	Reported Result	Reference	Comments
2,5-Dimethylfuran-3(2H)-one [13.119]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3 - 1000 µg/plate [1,2]	Negative	(Sokolowski, 2007)	All strains were negative. Study design complied with current GLP and OECD recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	33 - 5000 µg/plate [1,3]	Negative		
	Micronucleus Assay	Human peripheral blood lymphocytes	900 - 1120 µg/mL [1,6] 900 - 1120 µg/mL [4,7]	Negative	(Lloyd, 2011)	The MNBN cell frequencies in all treated cultures fell within the normal range. Complies with draft OECD Guideline 487 and GLP recommendations.
4-Acetyl-2,5-dimethylfuran-3(2H)-one [13.175]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32 - 5000 µg/plate [1,2]	Negative	(Bowen, 2011)	Evidence of toxicity was observed at 5000 µg/plate in all strains in the absence and presence of S-9. Study design complied with current GLP and OECD recommendations.
	Micronucleus Assay	Human peripheral blood lymphocytes	1000 - 1542 µg/mL [1,6] 400-950 µg/mL [1,7]	Equivocal	(Lloyd, 2012)	Study in compliance with GLP and OECD recommendations. Statistical significant increase, dose-related, in the presence of S9-mix at all three concentrations in a first experiment. Lower stastical significance at the two highest concentrations in an enlarged scoring, carried out with an unjustified approach. Mean MNBN cell frequencies fell within the historical control range with exception of a single replicate.

[1] With and without S9 metabolic activation.
[2] Plate incorporation method.
[3] Pre-incubation method.
[4] Without S9 metabolic activation. (not used).
[5] With S9 metabolic activation.(not used).
[6] 3-hour incubation with 21-hour recovery period.
[7] 24-hour incubation with no recovery period.

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ABBREVIATIONS

ADI	Acceptable Daily Intake
BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids Chemical Abstract Service
CHO	Chinese hamster ovary (cells)
CHL	Chinese Hamster Lung (cells)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EC	European Commission
EFFA	European Flavour and Fragrance Association
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ICH	International Conference on Harmonisation
ID	Identity
IOFI	International Organization of the Flavour Industry
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50%; Median lethal dose
MS	Mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
No	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative Structure-Activity Relationship
SCE	Sister Chromatid Exchange

SCF	Scientific Committee on Food
SMART	Somatic Mutation and Recombination Test
TAMDI	Theoretical Added Maximum Daily Intake
UDS	Unscheduled DNA Synthesis
WHO	World Health Organisation